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**“GENDER DIFFERENCES IN HUMAN SMOOTH MUSCLE CELLS  
AND RAT LIVER”**

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## Introduction

### *Sex and Gender*

The terms sex and gender are commonly used interchangeably, or are referred to a dichotomous variable. However, gender and sex indicate different aspects. Gender is a sociocultural pattern that sorts and organizes social relationships, social standards and values, appropriate roles for men and women <sup>1-3</sup>, while sex indicate the biological differences of the body. Thus sex-gender differences will reflect physiological distinctions between each sex, as well as environmental influences dictated by differences in the diet, life style, and exposure to environmental pollutants (e.g. chemicals, perfumes, cigarette smoke)<sup>3,4</sup>. Where appropriate, cultural and behavioral differences are highlighted and contrasted against those due to biological differences <sup>5</sup>. Moreover, sex and gender interact constantly and their interactions could be more complex than previously believed. For example, nongenetic transgenerational inheritance presents a conspicuous marked sexual dimorphism. Thus, sex appears to be an 'environmental' variable that includes cellular, metabolic, physiological, anatomical and even behavioural differences, either in girls or boys or between men and women; thus, it should be included in the experimental tasks.

Historically, men have been the investigators and the participants of health research but data arising from these studies have been extrapolated to represent the experiences of both sexes <sup>6</sup>. Nevertheless, it is indisputable that there are substantial biological and social differences in the lives of females and males. These differences could have important health consequences along lifespan.

The recognition of the differences and similarities can modify diagnosis and improve the prevention, the efficacy and the safety of the treatments <sup>3,7</sup>. All these goals require experimental and clinical studies in which the design and analysis are performed in a new perspective, in order to go beyond a rough evaluation of differences and similarities, including the fact that females are not a homogenous population. It should also be considered what happens to and around us. Thus, social relationships should be examined: phenotype inevitably depends on the interaction of the individual with the environment (stress response) <sup>8,9</sup>, on maternal behaviour (e.g. rat mothers behave differently versus female and male pups)<sup>10,11</sup>.

Sexual dimorphism starts in uterus and seems also to occur at a pregonadal stage <sup>12</sup>. It is well known that female fetus, that develops between male fetuses, enters puberty later and is more aggressive towards other females; whereas, a male fetus, that develops between two female fetuses, is less aggressive and has

less sexual activity<sup>13</sup>. Also, neonatal changes can perpetuate, in adulthood and in the successive generation, involving somatic epigenomic alterations, hypothalamic-pituitary-adrenal axis<sup>12,14-16</sup>. Considering that sex-gender “dimension” starts in a very early age, it is salient to investigate differences in cell coming from individuals at very early age such as the cells of the umbilical cord.

Beyond the phenotypic differences, males and females differ in health, life span, cognitive abilities<sup>17,18</sup>, in drug responses<sup>19</sup>, diseases such as anemia<sup>20</sup>, hypertension, coronary heart disease<sup>21-23</sup> and renal dysfunctions<sup>24-28</sup>.

The evolution of knowledge regarding sex-gender differences in coronary heart disease (CHD) has allowed us to highlight differences in terms of prevalence, onset of symptoms, and pathophysiology of CHD between men and women. Seung Hwan Han et al.<sup>21</sup> show that male patients were significantly younger than women, had lower levels of HDL cholesterol and the atherosclerotic plaques were larger and had more pronounced eccentricity. It has also been reported that the aggressive revascularization strategies do not lead to the same benefits in women than in men<sup>21,29,30</sup>. However, the mechanisms underlying these differences remain obscure. The clinical data and in vitro studies show that one of the first consequences of the outbreak of the atherosclerotic process is the remodeling of the vascular smooth muscle<sup>31</sup>.

The spindle-shaped VSMC derived from mesenchymal cells form the tunica media of the vessels. In mature animals, these cells are deeply specialized and participate in the regulation of blood vessel tone and/or diameter, and blood flow distribution. Contraction and dilatation are controlled by innervation for example through noradrenaline, by hormones, autocrine/paracrine agents such as angiotensin II (Ang II), leukotrienes<sup>32,33</sup>. Ang II is a multifunctional hormone that has pleiotropic effects on smooth vascular muscle causing vasoconstriction, proliferation of vascular smooth muscle cells (VSMC)<sup>34,35</sup>, cellular senescence, oxidative stress<sup>36-39</sup>, inflammation and apoptosis<sup>40,41</sup>. Ang II acts through at least two types of receptors: AT1 e AT2. The AT1 is seven transmembrane class of G-protein-coupled receptor that mediates the major cardiovascular effects of Ang II<sup>42</sup>. The human genome contains a unique gene coding for the AGTR1 receptor, localized in 3q24. Multiple alternatively spliced transcript variants have been reported for Ang II receptor, type 1 (AGTR1) gene. AGTR2 is located in Xq22-q23 and encodes AT2 receptor that belongs to the G-protein coupled receptor 1 family<sup>43,44</sup>. It is an integral membrane protein that is highly expressed in fetus, but scantily in adult tissues, except brain, adrenal medulla, and atretic ovary<sup>45</sup>. This receptor has been shown to mediate programmed cell death and this

apoptotic function may play an important role in developmental biology and pathophysiology<sup>46</sup>. All Human fibroblasts have an abundant expression of both AT1 and AT2 receptors, whereas VSMC express only AT1<sup>47</sup>. Leukotrienes are lipid mediators generated *de novo* from membrane-associated arachidonic acid which play a role in the control of bronchial<sup>48-50</sup> and vascular muscle where modulate proliferation, hypertrophy, hyperplasia, and remodeling of the arterial wall of vascular smooth muscle<sup>51</sup>. They activate cysteinyl leukotriene receptor 1 which is encoded by the CYSLTR1 gene<sup>48</sup>, located in Xq13.2-21.1. Noteworthy genes located in X chromosome for humans, are also located in X chromosome for every mammals or at least for those in Homologene database

([http://www.ncbi.nlm.nih.gov/sites/entrez?cmd=Retrieve&db=homologene&opt=HomoloGene&list\\_uids=4837-20172-28](http://www.ncbi.nlm.nih.gov/sites/entrez?cmd=Retrieve&db=homologene&opt=HomoloGene&list_uids=4837-20172-28)).

Indeed, while somatic chromosome are very arranged among species, this is not true for sexual ones which are very conserved<sup>52</sup>, probably due to the need to maintain a dosage compensation and simultaneously sex determining role<sup>53</sup>. Notably, VSMC appear to be, in the adult mammalian, cells with a high plastic properties, capable of run into major, reversible changes in morphology and gene expression in response to local stimuli both *in vivo* and *in vitro*<sup>31,54</sup> e.g. they can also differentiate into osteoblast-like cells at sites of atherosclerosis/arterial calcification<sup>55</sup>. VSMC possess receptors of sexual hormones, and the estrogen receptors diverge in male and female<sup>21,38,56-58</sup>. Nevertheless, only few studies have been focused on sex-gender differences in VSMC and they are performed in VSMC obtained from animals. The differences involve proliferation, cell death (apoptosis and autophagic process) and redox state<sup>59,60</sup>.

Human VSMC can be easily obtained from the umbilical cord which connects the developing embryo or fetus to the placenta<sup>61</sup> and in humans it normally contains two umbilical arteries and one umbilical vein, immersed in Wharton's jelly. The umbilical vein and arteries are twisted in a spiral with only a small amount of Wharton's jelly between them. This close association raises the possibility of a hemodynamic interaction between the arterial and venous vessels<sup>62</sup>. The umbilical veins transport oxygenated, nutrient-rich blood to the fetus from the placenta. Conversely, the umbilical artery transports the fetal deoxygenated, nutrient-depleted blood through back to the placenta<sup>63</sup>. Human VSMC obtained from the umbilical cord have been already used in experimental settings that study the physiology and pathology of cardiovascular system<sup>64</sup>.

As already mentioned, the androgen receptor (AR) is present in VSMC and belongs to nuclear receptor subfamily 3, group C, member 4. The human gene

the AR has been localized to Xq11-12. The AR regulates directly and indirectly gene expression<sup>65-69</sup>. The activation of AR exerts controversial effects on VSMC. Hashimura et al. found that androgens stimulates an increase in GAG length and the binding capacity to LDL in VSMC cultures from mammary arteries<sup>67</sup>. On the other hand, Somjen et al.<sup>68</sup> found that the effect is dose dependent, low concentration increases and decrease VSMC proliferation, respectively. In humans, low plasma testosterone level is associated with advanced atherosclerosis<sup>65</sup> suggesting that androgens play a role in cardiovascular function.

More information are available on the role of estrogen receptors (ER) in VSMC. Estrogens exert their action through different types of receptors<sup>70-72</sup>. Two classes of estrogen receptor exist: ER alpha and beta, which are member of the nuclear hormone family of intracellular receptors, and the estrogen G protein-coupled receptor GPR30 (GPER), which is a G protein-coupled receptor<sup>73,74</sup>.

ER $\alpha$  and ER $\beta$  are encoded by a separate gene (ESR1 and ESR2, respectively) which show significant overall sequence homology, and both are composed of five domains. Ligands may differ in their affinity for alpha and beta isoforms of the ER<sup>75</sup>. Additionally, the presence of different ER combinations may be the cause different response to various ligands<sup>76</sup>. The ratio of  $\alpha$ - to  $\beta$ - subtype concentration has been proposed to play a role in certain diseases<sup>75</sup>.

To make this situation even more complicated there is at least another nuclear estrogen receptor: Estrogen-related receptor alpha (ERR- $\alpha$ ), also known as NR3B1 (nuclear receptor subfamily 3, group B, member 1). This is a nuclear receptor encoded by the ESRRA gene that is closely related to the estrogen receptor alpha and in humans is located in 11q13. The protein encoded acts as a site-specific transcription regulator<sup>77,78</sup>. This protein has been also shown to interact with estrogen and the transcription factor TFIIB by direct protein-protein contact. The binding and regulatory activities of this protein have been demonstrated in the regulation of a variety of genes including lactoferrin, osteopontin, medium-chain acyl coenzyme A dehydrogenase (MCAD) and thyroid hormone receptor genes<sup>79</sup>. Finally, the ERR alpha/PGC1alpha complex is a regulator of energy metabolism (STRING DATABASE). Recently it has also be individuated another ER namely GPER. This receptors is a specific membrane receptor and induce rapid nongenomic effects even in the vessels<sup>80</sup>. Its activation rapidly activates a series of signaling pathways in the cell, such as MAPK, cAMP, PI3K/Akt cascades and calcium current<sup>81-86</sup>.

ER $\alpha$  and ER $\beta$  and GPR30 affect cardiovascular function<sup>74,87-92</sup>. Nevertheless, there are still controversial aspects in the distribution of the expression of ER subtypes between the components of human cardiovascular system<sup>93</sup>.

Many sex-gender differences has been also described in liver. The most studied differences involve drug metabolism<sup>3</sup>. Actually, it is known that both phase 1 and phase 2 enzymes are sexually divergent and they could explain numerous differences in the drug response. Genetic polymorphisms<sup>94</sup>, the personal history of exposure to drugs and environmental chemicals<sup>95</sup>, dietary factors<sup>96,97</sup>, pregnancy<sup>98</sup>, diseased states<sup>99</sup>, epigenetic factors<sup>100</sup>, age and sex-gender<sup>101</sup> affect the activity of drug-metabolizing enzymes. Sex-gender differences involve CYP enzymes<sup>3</sup>, sulfotransferases<sup>102</sup>, glutathione transferases<sup>103</sup> altering the bioavailability and clearance of xenobiotics<sup>3</sup> in male and in female.

GSH plays a central role in redox state. It can directly scavenge reactive oxygen species or act as substrate of enzyme such as glutathione transferases. These enzymes, as already mentioned, are sexual divergent and are involved in drug metabolism. Actually, it is less known whether the synthesis of GSH is sex-gender dependent. The rate limiting step in the synthesis of GSH is the glutamylcysteine ligase (GCL)<sup>104</sup>. The ligase incorporate cysteine in the GSH molecule, while a part of cysteine is degraded into sulfate, H<sub>2</sub>S and taurine<sup>105,106</sup>. Notably, the enzyme is also involved in hepatic paracetamol toxicity<sup>107,108</sup> participating to redox state and xenobiotic metabolism<sup>109</sup>.

### ***Genetically based differences***

Genetic diversity in humanity has been estimated at about one nucleotide to 300<sup>110</sup> so every individual has a combination of genetic variants with a total effect that varies widely from one individual to another.

Female mammals cells possess two copies of the X chromosome but, generally speaking, they do not synthesize twice as much of those proteins whose gene is located in the X chromosome because one of the X chromosome is inactivated through a process called lionization, the A Barr body (named after discoverer Murray Barr)<sup>111,112</sup>. In 1961, Mary Lyon shows that process occurs early in embryonic development and in mammals it occurs at random<sup>113</sup>. Later, Beutler, studying heterozygous females for Glucose-6-phosphate dehydrogenase (G6PD) deficiency discovered the female mosaicism<sup>114</sup>.

The inactive X chromosome (Xi) when compared to the active X chromosome (Xa) has high levels of DNA methylation, low levels of histone acetylation, low



levels of histone H3 lysine-4 methylation, and high levels of histone H3 lysine-9 methylation, all of which are associated with gene silencing<sup>115</sup>. Additionally, a histone variant called macroH2A (H2AFY) is exclusively found on nucleosomes along the Xi<sup>116</sup>. Xi does not express the majority of its genes but not the whole barr body is inactive<sup>117</sup>. Talebizadeh et al.<sup>118</sup> found, in a published dataset of multiple normal human tissues<sup>119</sup>, that on average 5.1 and 4.9% of genes showed higher expression in females compared with 7.4 and 7.9% in males, respectively, for X-linked and autosomal genes<sup>118</sup>.

### ***Epigenetics***

Living beings need for a continuous adaptation to the environment, in other words, environment modulates the expression of genes and gene networks through fine adjustments based on the conditions of life, and on molecules with which they come into contact. Such molecules come not only from food and drugs, but also from botanical remedies, dietary supplements, drinks and airborne substances (e.g. alcohol and environmental pollutants like tobacco smoke and metals)<sup>120,121</sup>. The influence of environment could generate different response in male or female bodies. All this falls under the action of epigenetic mechanisms<sup>122</sup>

### ***Hormonal Differences***

They are briefly mentioned when AR and ER have been discussed.

### ***Autophagic, lysosomal marker and mTOR***

Macroautophagy (hereafter called autophagy) is a process during which the regulated turnover of cellular constituents happens through lysosomal machinery<sup>123</sup>. Autophagy delivers cytoplasmatic cargo to the lysosomes through a double-wall vesicles called autophagosomes which fuse with lysosomes to form an autophagolysosome. This process, which exists in all eukaryotic cells, is tightly controlled, and in extreme cases can result in cell death<sup>124</sup>. For example, autophagy is fundamental during oocyte-to embryo transition<sup>125,126</sup>, in starvation autophagic cells are frequently observed in certain disease such neurodegenerative disorders like Alzheimer's and Parkinson's disease<sup>127</sup>, in liver failure, hepatitis, liver cancer and steatosis<sup>128</sup>. In addition, alterations in autophagic process seem to be implicated in hypertensive heart disease and heart failure<sup>129,130</sup> in ischemia/reperfusion<sup>131,132</sup>. So it is plausible that autophagy

is not only an adaptive response to nutrient limitation but also a mechanism for cell suicide and a mechanism for cell homeostasis<sup>133,134</sup>. Many proteins are involved in the autophagic processes. Beclin 1 is involved in the early steps of autophagic vesicle formation and it is a haplo-insufficient tumor suppressor gene<sup>135,136</sup>. Also microtubule-associated protein light chain 3 (LC3)<sup>137</sup> involved in autophagy. Upon induction of autophagy, native LC3 (LC3 I) is lipidated by conjugation to phosphatidylethanolamine (becoming LC3II) and targeted to autophagic membranes. Therefore, changes in LC3 expression and localization have been used to measure autophagy. Thus, the LC3-II/LC3-I is a good early marker for autophagy.

Actually, it is not known whether autophagy present sexual dimorphism. However few papers suggest that it could occur. Du et al.<sup>138</sup> found that nutrient restriction increased autophagosome formation in males rat neurons more than in females. It has been shown that spontaneous and induced autophagy diverges in rat VSMC<sup>59</sup> obtained from male and female animals. Data on organ are missing therefore one of the aim of this thesis was to examine autophagy in different rat organs studying two autophagic biomarkers: Beclin-1 which is required for the initiation of the formation of the autophagosome<sup>135,139,140</sup> and microtubule-associated protein light chain 3 (LC3)<sup>137</sup>.

Furthermore, we also evaluated an opposite regulator in autophagy induction: the kinase mTOR, which, when activated, inhibits autophagy<sup>139</sup> and oxidation of proteins and lipids because redox state is an important event for induction of autophagy. In view of the role of lysosomes in the autophagy<sup>139</sup>, we also measured LAMP-1, a constitutive protein of lysosomal membrane.

## Methods

### *Animals*

18 male and 18 female Sprague–Dawley rats (7 weeks old) were purchased from Harlan (Italy) and housed 2–3 per cage. Rats were maintained on a 12 h light/dark cycle and were allowed food and water ad libitum till the sacrifice. The experimental protocols were carried out in accordance with Italian law (DL 116, 1992) and the NIH principles of laboratory animal care (NIH 80-33, revised 1996). All experimental procedures were approved by the Department of Veterinary Public Health, Food Security and Collegial Organs for the Protection of Health (Ministry of Health) as requested by Italian law. As it was impossible to have males and females at the same age of the same weight, we decided to perform experiments in samples obtained from male and female animals of the same age. Animals were euthanized by decapitation, then thoracic and abdominal cavities were opened to access the liver, which was rapidly removed, washed, and divided into two weighed parts, one part was homogenized in ice cold PBS. Homogenates were stored at  $-80^{\circ}\text{C}$  and analyzed within 1 month. Part of the liver was used for immunohistochemical staining, after fixation in 4% paraformaldehyde and paraffined.

### *Thiol measurement*

Thiols were measured according to Zinellu et al.<sup>141</sup>. For thiol analysis, 100  $\mu\text{l}$  of standard or samples were mixed with 10  $\mu\text{l}$  of TBP (10%), vortexed for 30 s and subsequently incubated at  $4^{\circ}\text{C}$ . After 10 min 100  $\mu\text{l}$  of 10% trichloroacetic acid (TCA) were added and the mixture. After vortexing for 10 s, the reaction mixture was centrifuged for 10 min at  $3000 \times g$ . 100  $\mu\text{l}$  of supernatant were mixed with 100  $\mu\text{l}$  of 300 mmol/l  $\text{Na}_3\text{PO}_4$  at pH 12.5 and with 25  $\mu\text{L}$  of 5-IAF (4.1 mmol/l), and subsequently incubated at room temperature (RT) for 10 min. The mix was diluted 1/100 before being injected for capillary electrophoresis with a laser-induced fluorescence detector <sup>141</sup>.

### *Taurine detection*

50  $\mu\text{l}$  of hepatic homogenate were mixed with 50  $\mu\text{l}$  IS homocysteic acid (200  $\mu\text{mol/l}$ ) then were 100  $\mu\text{l}$  of TCA (10%) added to precipitate total proteins. After centrifugation at  $3000 \times g$  for 5 min, 10  $\mu\text{l}$  of supernatant were mixed with 90  $\mu\text{l}$  of 100 mmol/l  $\text{Na}_2\text{HPO}_4$ , pH 9.5 and 11  $\mu\text{l}$  of 15 mmol/l FITC. After 20 min incubation time at  $100^{\circ}\text{C}$ , samples were diluted 100-fold and injected into a capillary electrophoresis apparatus. Analysis was performed in an uncoated

fused silica capillary, 75  $\mu\text{m}$  I.D. and 47 cm length (40 cm to the detection window), injecting 18 nl of sample. Separation was carried out in a 20 mmol/l tribasic sodium phosphate buffer, pH 11.8, 23 °C at normal polarity 22 kV<sup>142</sup>.

### ***Measurement of H<sub>2</sub>S***

H<sub>2</sub>S was determined using the method described in Hua et al. (2009) with slight modifications. In brief, 1 ml of liver homogenate was mixed with 100  $\mu\text{l}$  of zinc acetate (1%, w/v) and incubated at 37 °C for 10 min. After proteins precipitation with 20% TCA, supernatant was collected by centrifugation (14,000 rpm  $\times$  20 min, 4 °C) and diluted twice with distilled water and incubated with N,N-dimethyl-p-phenylenediamine sulfate (2 g/l) and ferric chloride (FeCl<sub>3</sub>, 3 g/l) in HCl 6 N for 20 min at room temperature [RT] followed by a centrifugation at 14,000 rpm for 5 min at 4 °C. The absorbance of the resulting solution was measured at 670 nm and H<sub>2</sub>S concentration was calculated using a calibration curve of sodium hydrosulfide in water (100–3.125  $\mu\text{M}$ ). H<sub>2</sub>S blanks were obtained incubating the supernatant with FeCl<sub>3</sub> only. Results are expressed as nmol/mg protein.

### ***Malondialdehyde (MDA) detection***

MDA levels were measured according to Esterbauer and Cheeseman (Esterbauer and Cheeseman, 1990) with slight modifications. 200  $\mu\text{l}$  of tissue homogenate were mixed with 200  $\mu\text{l}$  of dilute acetic acid (1:3 in H<sub>2</sub>O). 150  $\mu\text{l}$  of 10% sodium dodecylsulphate (SDS), to easily precipitate the proteins, and 200  $\mu\text{l}$  of Tris-HCl 50 mM were added. The solution was kept in incubation for 10 min at RT and then 500  $\mu\text{l}$  of a solution of thiobarbituric acid [0.75% in dilute acetic acid and NaOH 1N (1:1)] were added. The mixture was boiled for 60 min, quenched in ice (10 min), added with 400  $\mu\text{l}$  of acetic acid (1:3 in H<sub>2</sub>O) and centrifuged at 4°C at 13000 rpm for 20 min. The quantification was performed spectrophotometrically at 532 nm by measuring the absorbance produced by the sample. Standards of MDA at known concentration (5, 10, 25, 50  $\mu\text{M}$ ) were used to construct the calibration curve.

### ***Assay of carbonyls***

Total amount of carbonylated proteins was quantified as described by Fagan et al<sup>143</sup> with some modifications. In brief, 10  $\mu\text{l}$  of tissue homogenate were washed with 10 volumes of acetone-HCl (30:1); the pellet resuspended in 100  $\mu\text{l}$  of PBS and 900  $\mu\text{l}$  of 2,4-dinitrophenylhydrazine (DNPH) (10 mM in HCl 2 N) were added and incubated for 1 h at RT, with occasionally mixing. Blanks were

incubated with HCl 2 N only. Proteins were precipitated with 20% trichloroacetic acid (TCA), collected by centrifugation (10 min, 15000 rpm, 4°C) and washed with 10% TCA. To remove unreacted DNPH, samples were washed three times with 1 ml mixture of ethyl acetate-ethanol 1:1 and then the protein pellet was re-suspended in guanidine hydrochloride (6 M in HCl 2 N) at RT. The absorbance was recorded at 370 nm and the carbonyl concentration was calculated using the extinction molar coefficient of DNPH ( $\epsilon = 22000$ ) after subtracting blank's absorbance. Carbonyls concentration was normalized using the protein concentration of blanks measured at 280 nm.

***Western blot GCL, actin,  $\beta$ -tubulin, glyceraldehyde- 3-phosphate dehydrogenase (GAPDH) in liver homogenates.***

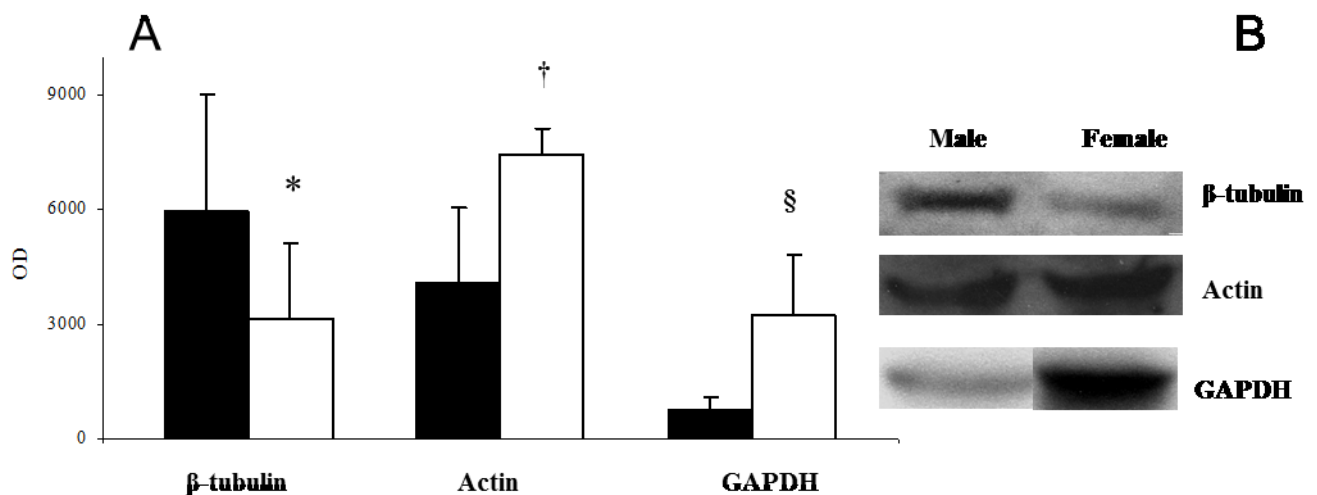
80  $\mu$ g of protein for each sample were loaded. The reaction was carried out using anti-actin IgG fraction of antiserum developed in the rabbit (A5060, Sigma-Aldrich, Italy), a polyclonal rabbit anti- $\beta$ -tubulin (2146S, Cell Signalling Technology, DBA Italia, Italy), a monoclonal rabbit anti-GAPDH (2118S, Cell Signalling Technology, DBA Italia, Italy) and a polyclonal rabbit anti-GCLc (sc-2275S, Cell Signalling Technology, DBA Italia, Italy). Specific protein was detected by chemiluminescence reaction (LumiGLO, Cell Signalling Technology, DBA Italia, Italy), followed by densitometric analysis of immunoblot by dedicated software (Labworks). Western blot analysis showed that total actin and GAPDH expression were significantly higher in females than in males ( $P = 0.048$  and  $P = 0.017$ , respectively; data not shown) whereas  $\beta$ -tubulin expression was significantly lower in female than in male tissue ( $P = 0.006$ ; data not shown) and therefore we were unable to normalize Western blot data for these proteins. The reported differences were in line with those found by others.

80  $\mu$ g proteins were resolved by SDS-PAGE (4-15%) at 100 V for 1 h at RT and then electrophoretically transferred to PVDF membrane for 1 h at 250 mA and 4 °C using a mini-PROTEAN tetra-Cell system (Bio-Rad Italia, Segrate, Italy). The PVDV membrane was treated with 5% w/v skim milk in Tris buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.2) at RT for 2 h and then probed overnight at 4°C with specific polyclonal antibodies, (actin (1:1000),  $\beta$ -tubulin (1:1000), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:1000), mTOR (1:1000), Beclin-1 (1:1000), LAMP-1 (1:1000) and LC3 which reacts with both LC3-I and LC3-II (1:500). Antibodies were obtained from Cell Signalling Technology (DBA Italia, Segrate, Italy) with the exception of anti-LC3 antibody that was purchased from Eppendorf Italia (Milan, Italy).

After washing in Tris buffer, blots were incubated for 1 h with horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000; Cell Signalling Technology, DBA Italia, Segrate, Italy). Specific proteins were detected by chemiluminescence reaction (Luminata<sup>TM</sup> Classic Western Blot HRP substrate, Millipore, Vimodrone, Italy), followed by densitometric analysis of immunoblot by dedicated software (Labworks).

### ***Selection of housekeeping protein for normalization of western blot data***

Previously, in rat liver, it was shown that some housekeeping proteins most frequently used to normalize Western Blot were sexually divergent<sup>144</sup>. Therefore, we evaluated actin,  $\beta$ -tubulin and GAPDH, in livers to select the protein for normalization. Fig. 1 evidenced that all three proteins were sexually divergent in the livers. Consequentially, data from liver were normalized using total proteins, which did not diverge between males and females.



**Fig. 1.** Densitometric analysis (panel A) and representative Western blot (panel B) of  $\beta$ -tubulin, actin and GAPDH expression in livers obtained from male and female rats. Data for densitometric analysis are reported as mean  $\pm$ SD of 5 independent samples per group. \* P=0.006; † P=0.048; § P=0.017.

### ***Immunohistochemistry of GCL***

Immunohistochemistry was performed using a polyclonal rabbit anti-GCLc (Cell Signalling Technology, DBA Italia, Italy) and the LSAB+ System-HRP Kit (Dako, Italy). For antigen retrieval, 3- $\mu$ m thick sections were heated at intermediate power in a conventional microwave oven in citrate buffer (10 mM, pH 6) for 10 min. The slides were blocked with 3% hydrogen peroxide for 20 min and incubated with primary antibody (1:50) in PBS/BSA 1% overnight at 4°C, washed, incubated with biotinylated goat anti-rabbit IgG in PBS for 30 min, washed, incubated with streptavidin-HRP reagent for 30 min, washed, and incubated with diaminobenzidine substrate for 2 min. Finally, slides were counterstained with hematoxylin, dehydrated in graded ethanol washes and washed in xylene. Liver sections were examined in a blinded manner under light microscopy (DM4000B, Leica).

### ***Immunofluorescence microscopy of LAMP-1 and LC3***

Immunofluorescence microscopy analyses were carried out in livers. A double labeling of LAMP-1 and LC3 was performed. The first is a lysosome-associated membrane protein involved in the maintenance of lysosome acidity and is used to mark lysosomal localization. The second is a microtubule-associated protein, present in the cells in two forms: soluble form (LC3-I) and autophagosome-associated form (LC3-II). LC3-II and LAMP-1 co-localization indicates autophagolysosomes formation (Trincheri et al., 2007). 5  $\mu$ m thick sections were deparaffinized by washing in xylene and rehydrated through a graded series of ethanol. After redistilled water washings, sections were incubated with polyclonal anti-LC3 (Eppendorf Italia, Milan, Italy), which reacts with both LC3-I and LC3-II, and monoclonal anti-LAMP-1 (Santa Cruz Biotechnology, California, USA) for 30 min at 37 °C. After extensive washing, sections were exposed to the appropriate TRITC- and FITC-conjugated secondary antibody (Sigma-Aldrich, Milan, Italy) for 30 min at 37 °C. For nuclear staining, the dye Hoechst 33258 (Sigma-Aldrich, Milan, Italy) was used. Samples were examined with an Olympus BX51 microscope and immunofluorescence analyses were carried out by intensified video microscopy (IVM) by a Charge-coupled device camera (Carl Zeiss, Oberkochen, Germany).

## ***Cell isolation and cultivation***

### ***Source of materials and selection of donors***

Umbilical cords were provided by the Department of Pharmacology, Gynecology and Obstetrics, University of Sassari. We selected umbilical cord coming from human healthy male and female newborns (total female =16 , total male =16), which were vaginal delivered from Caucasian, healthy, non-obese and non-smoking mothers that were drug free with exception of folic acid.

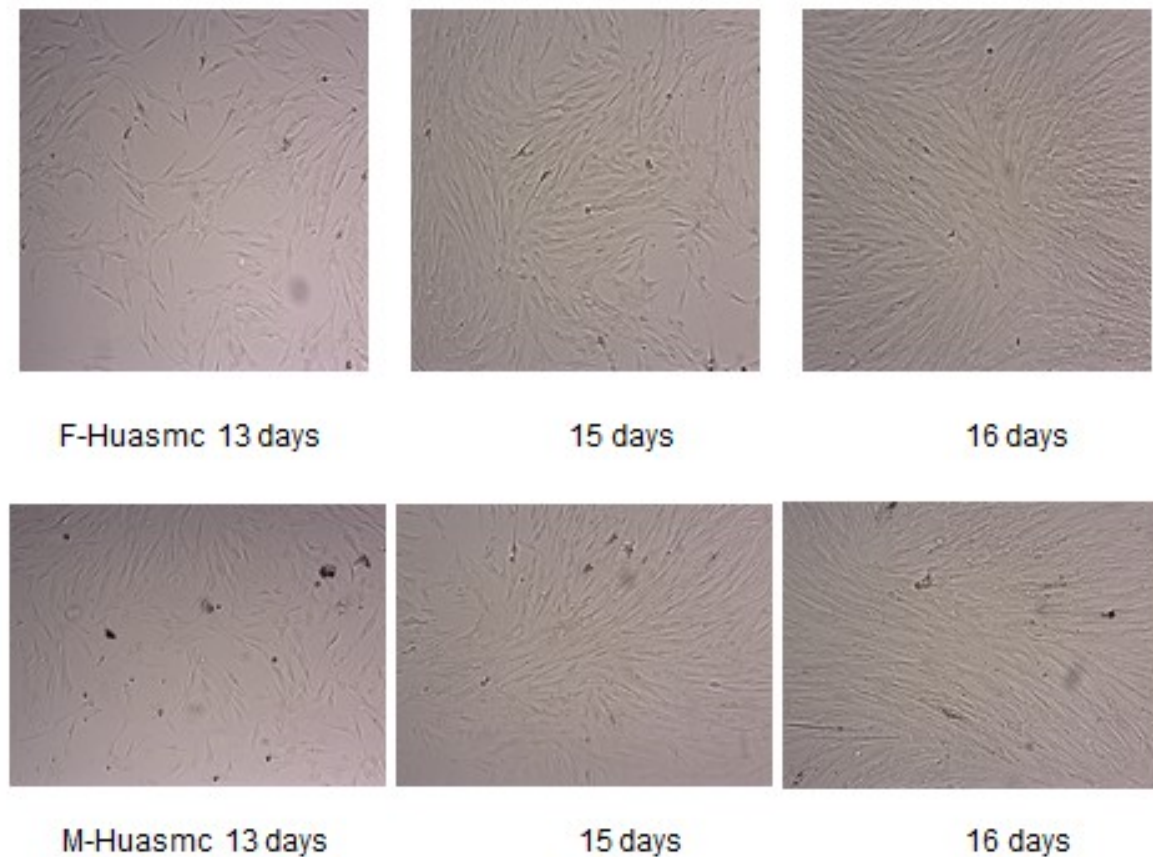
Informed consent was obtained from all subjects donating cords in accordance with the Declaration of Helsinki.

### ***Isolation of Human Umbilical Artery Smooth Muscle Cells: HUASMC***

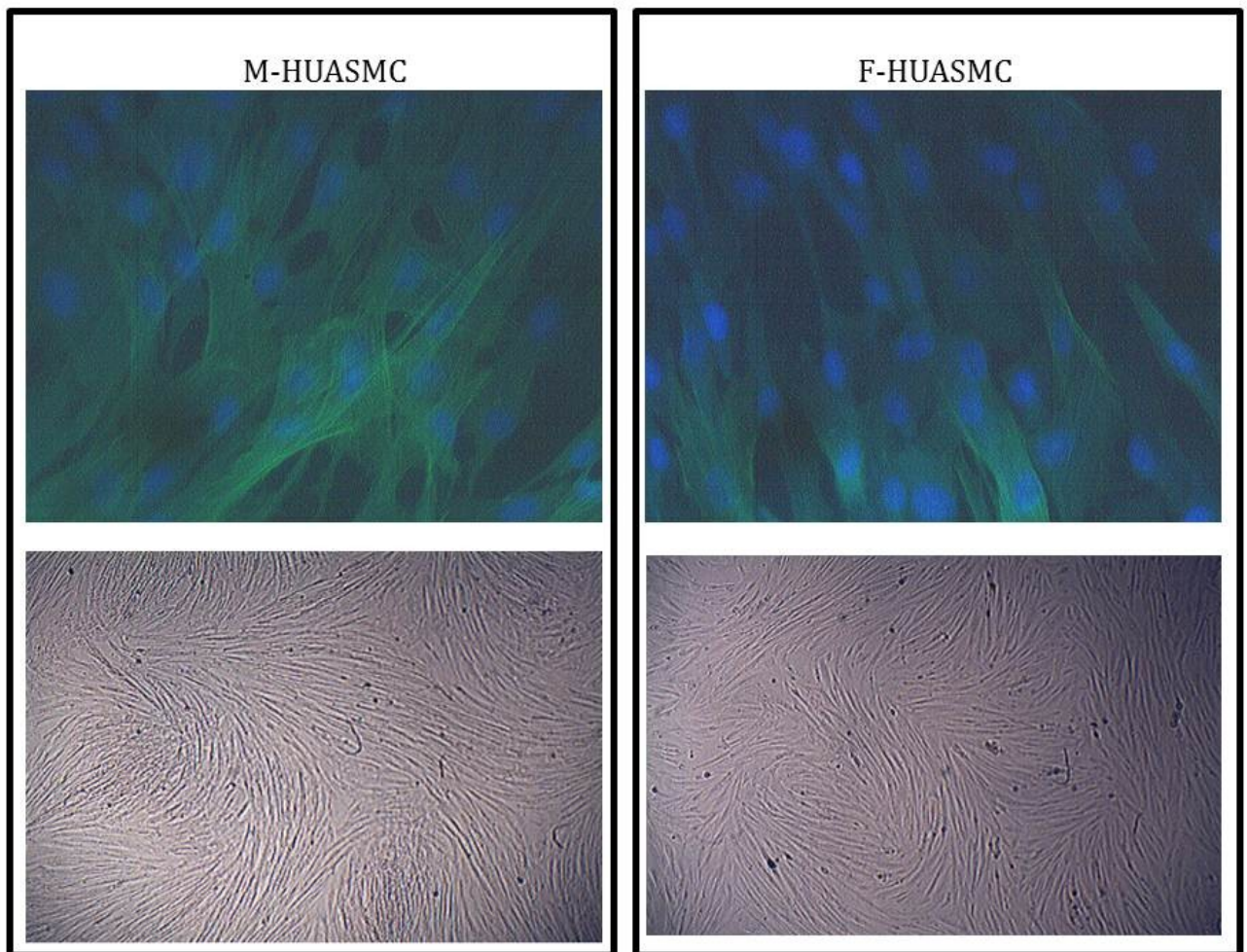
Various methods of isolation are described in the literature<sup>145-147</sup>, however in my hand, none was satisfactory in term of the cells recovery, the purity and cellular suffering. So I proceeded to some modifications. Precisely, cords were washed externally in PBS (Sigma-Aldrich, Saint Louis, USA) immersed in PBS and cut into segments not longer than 8 cm to avoid supercoilings. Then, cords were longitudinally opened following the path of the arteries and were separated from connective gelatinous (Wharton's jelly) and adventitia. The blood contained in the arteries were drained from the ends, and the segments were incubated at 37°C for 45 'in 5 ml of a solution 800 U/ml of collagenase from *Clostridium histoliticum* (Sigma-Aldrich, Saint Louis, USA) dissolved in DMEM medium with Glutamax (Gibco). Then the suspension of segments of artery was inverted in a cell culture grade Petri dish. After adding further 15 ml of PBS, segments were grasped with tweezers at one end and externally scraped along the entire length with another, so as to facilitate the suspension of HUASMC. The complex thus obtained is then diluted with PBS to the extent of 100 ml each 10 cm in length of the cord to reduce the viscosity due to the extracellular matrix solubilized by enzymatic action. Macroscopic debris are discarded and only the aqueous phase is centrifuged (at 1000G for 15 min). The pellet were resuspended in 15ml of 20% FBS DMEM medium with glutamax, (Sigma-Aldrich, Saint Louis, USA), with 1% antibiotic / antimycotic (Sigma-Aldrich, Saint Louis, USA), and seeded in gelatin coated culture flasks (Sigma-Aldrich, Saint Louis, USA;1% in PBS, dried) to the extent of 75 cm<sup>2</sup> of adhesion surface each 20cm departure umbilical cord and kept at 37°C in an atmosphere of 95% relative humidity at 5% CO<sub>2</sub>. After six days, the flask were gently horizontally shaken to resuspend the thin extracellular sediment layer, the old medium was aspirated and the culture washed with PBS, then fresh medium



were added. Upon reaching confluence the p0 cells were trypsinized and reseeded at 75% confluence (Fig. 2).



**Fig.2.** Huasmc proliferation and transition from secretory to contractile phenotype. That transition become definitely evident in two or three days at 100% of confluency.



**Fig.3.** Female and Male HUASMC P1 cultures. The picture show the morphology, alpha smooth muscle actin and the confluence of HUASMC.

***Cellular characterization by alpha smooth muscle actin-specific immunofluorescence***

The cells were seeded at a density of 35000 cells/cm<sup>2</sup> in chamber slide. 48 hours later, after settling down, cells were washed with PBS and then fixed in 4% PFA in PBS for 5 minutes at RT. After a further wash in PBS cell layer was fixed in cold methanol for 1 minute at room temperature to facilitate the permeabilization. The cells, washed again with PBS were treated with blocking solution (PBS with the addition of 4% BSA and 0.1% Triton X-100) for 10 minutes and incubated with the primary antibody (alpha smooth muscle actin-

specific dilution 1:400 in blocking solution) for 60 minutes at RT. After 2 washes with PBS we proceed with the incubation with the secondary antibody (diluted from 1:64 to 1:100), conjugated with the fluorescent probe, for 30 minutes in the dark. After further washing in PBS, cell layer was stained with DAPI, (1ug/ml) for 4 minutes in the dark, to label the nuclei. After a final wash in PBS, the slides are mounted with mounting Medium for fluorescence (Fig.3).

### ***RNA extraction and reverse transcription***

P1 cultures of HUASMC, were washed with PBS and total RNA extracted by the use of silica column/buffer guanidinium and DNA digestion “in-column” by means of the commercial kits "RNeasy mini prep" and the enzyme DNase-RNase free both from Qiagen according to the manufacturer's instructions. The extracted RNA was quantified by spectrophotometric reading at 260 nm and A280 nm to assay the protein contamination. 1ug of RNA for each sample was converted into cDNA by the “High Capacity cDNA Reverse Transcription Kit”, purchased from Applied Biosystems, according to the indication of the producer. Random RNA samples were always retrotranscribed with H<sub>2</sub>O instead of reverse transcriptase (rt-blanks) to check for genomic amplification during real time PCR.

### ***Real time PCR***

The Real time PCR reactions were carried out at first in triplicate then in duplicate to save sample, given that the "variability between the technical replicates" was too small to be relevant. 20ng of cDNA were used as template in a total reaction volume of 10µl. We used the TaqMan probes according to the manufacturer's instructions. Taqman probes were chosen checking the existence of synonyms in databases and specificity compared to pseudogenes and isoforms. where possible have been selected probes straddling exon junctions. Rt-PCR results for rt-blanks were always negative given that treatment with DNase during RNA extraction prevented any genomic contamination. Two housekeeping genes were used to normalize real time PCR results: the gene that encode the ribosomal protein l30 (Rpl30) a protein that is a component of the ribosomal 60S subunit<sup>148</sup> and the gene that encode β2 microglobulin, a component of MHC class I molecule<sup>149</sup>, that is a good candidate as a housekeeping gene for normalization<sup>150</sup>.

***Statistical analysis***

Each sample was determined in duplicate and statistical analysis of data was done by comparing parameters obtained from male and female rat livers. Continuous parametric variables were analyzed using the Student t-test. Non-parametric variables were compared with the Mann-Whitney rank test. For all tests a P-value  $\leq 0.05$  was considered as statistically significant. The association of strength between pairs of variables was calculated by the Spearman Product Moment Correlation coefficient or the Pearson Product Moment Correlation coefficient using SigmaStat software.

For statistical analysis of real time data Relative expression software tool (REST©) were used, with 2000 iterations.

## Results

### *Sex-gender differences in livers obtained from male and female rats*

#### ***Weight of animals and liver total protein content***

7 weeks old male and female rats were weighted and as expected, females were significantly smaller ( $P < 0.001$ ) in comparison with males (Table 1). After the sacrifices, total liver was assayed and they did not differ between sexes.

**Table 1. Body weight and total liver protein content of male and female rats.**

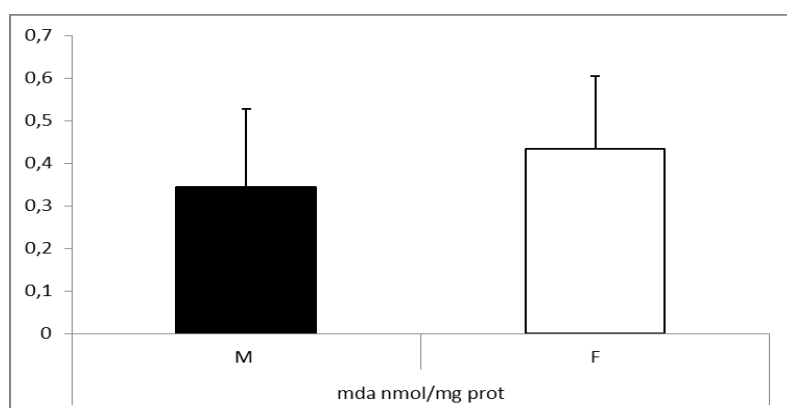
	Male	Female	P
Body weight (g)	223.3 $\pm$ 16.8	190.0 $\pm$ 10.87	<0.001
Total liver protein (mg protein/mg tissue)	0.16 $\pm$ 0.04	0.14 $\pm$ 0.04	NS

Data are reported as mean  $\pm$  SD of 18 independent animals per group.

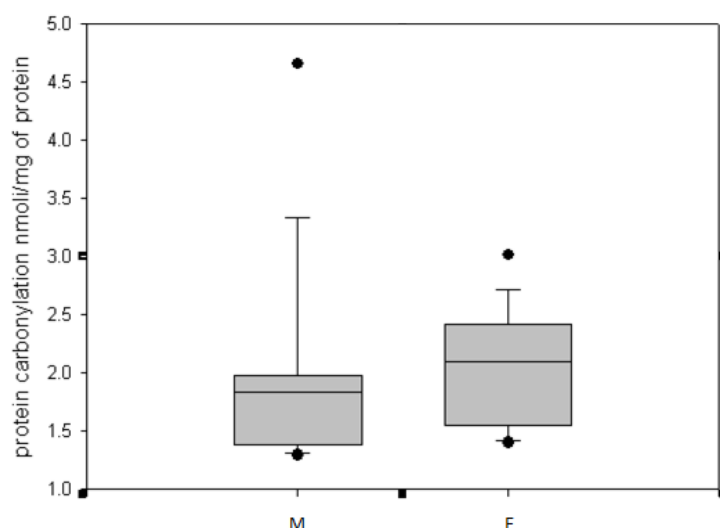
As shown in the material and method section, we did not find any valid housekeeping protein for a solid normalization between the two sexes. So we proceeded to normalize data on sample total protein content per mg of hepatic tissue.

#### ***Biomarkers of oxidative stress: MDA and protein carbonylation***

The redox state can be subject to sexual dimorphism<sup>60</sup>. We evaluated two oxidative stress markers MDA, a marker of lipid peroxidation<sup>151</sup>, and protein carbonylation<sup>152</sup>. Both MDA and protein carbonyls did not diverge in the liver of male and female rats (Fig. 4 and 5).



**Fig. 4.** MDA levels (data are reported as mean  $\pm$  SD of 18 independent animals per group).



**Fig. 5.** Protein carbonylation levels, 18 independent animals per group. Boxes represents the interquartile range, or the middle 50% of observations. The line represents the median. Whiskers represent the 10<sup>th</sup> and 90<sup>th</sup> percentiles, dots represent the minimum and maximum observations.

### ***Liver levels of some noteworthy thiol compounds***

In view of the importance of GSH in the metabolism of drugs we examined GSH and its precursors. GSH did not vary significantly between sexes but remarkably, L-cysteine was significantly higher in females than in males (Table 2), however precursor of cysteine, L-methionine, was not divergent between sexes (Table 2). L-cysteine metabolites, taurine and H<sub>2</sub>S was also detected because both have

important physiological functions. Briefly, taurine acts as antioxidant and is an endogenous hypochlorous acid scavenger<sup>153–155</sup>, while H<sub>2</sub>S participate in gastrointestinal mucosal defense and repair<sup>156</sup>, and regulation of vascular function<sup>157–163</sup>. Although we did not find any significant differences in taurine, H<sub>2</sub>S was significantly lower in female liver than in male liver (Table 2).

**Table 2. Levels of some thiol compounds (nmol/mg prot) in liver from male and female rats**

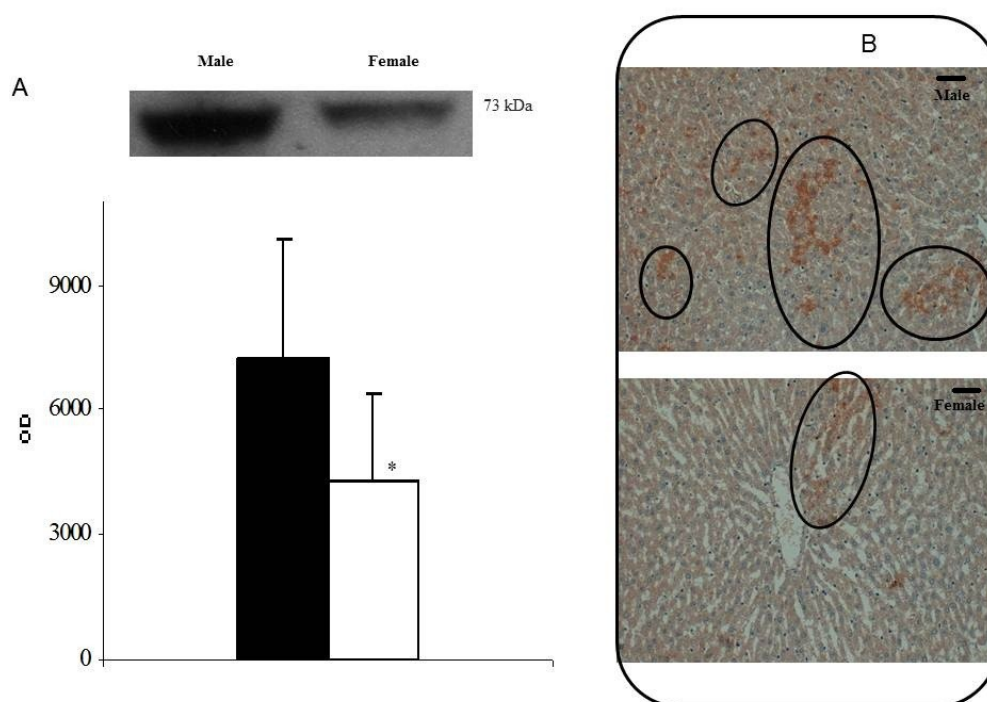
	Male liver	Female Liver	P
GSH	15.2 (10.64–37.76)	13.2 (5.63–38.54)	NS
L -Cysteine	2.39 (1.32–5.03)	4.22 (2.03–7.99)	0.007
L -Methionine	4.93 ± 1.20	5.49 ± 1.79	NS
Taurine	38.7 ± 14.0	35.2 ± 14.6	NS
H <sub>2</sub> S	0.22 ± 0.04	0.18 ± 0.04	0.016

Data are reported as mean ± SD of at least 18 independent animals per group when normally distributed and as median (range) if they have a non-Gaussian distribution.

### ***Expression of GCL, the key enzyme in GSH synthesis***

GCL was significantly lower (41%) in females than in males (P = 0.002) according to Western blot analysis (**Fig. 6.**, panel A). The biochemical data was confirmed by immunohistochemistry staining. Immunohistochemistry image showed smaller areas of positive staining (indicative of the different distribution of the enzyme) in paraffin sections obtained from female livers (**Fig. 6**, panel B) in comparison with paraffin sections obtained from male livers.





**Fig. 6.** Densitometric analysis, and representative Western blot of GCL expression (panel A) and a representative light microscopy demonstrating immunohistochemical staining of GCL (panel B) as observed in male and female rat livers. Data for densitometric analysis are reported as mean  $\pm$  SD of 7 independent samples per group. \* $P = 0.002$ . Ellipses indicate positively stained areas (brown cells). Images were captured at 20 $\times$  (bar = 50  $\mu$ m) of the picture.

### **Correlations**

Interestingly, in our findings, both in males and in females GSH and MDA ( $-0.785$ ,  $P < 0.001$ ;  $-0.554$   $P = 0.017$ , respectively) were inversely related, also GSH and protein carbonylation were negatively correlated both in males ( $-0.598$   $P < 0.001$ ) and in females ( $-0.43$ ;  $P = 0.0728$ ) even if P-value did not reach statistical significance in females, while L-cysteine and GSH ( $0.820$ ,  $P < 0.001$ ) such as L-cysteine and L-methionine ( $0.723$ ,  $P < 0.001$ ), and L-cysteine and taurine ( $0.554$ ,  $P = 0.017$ ) were positively associated only in males. GSH and taurine, L-cysteine and  $H_2S$  were not associated in both sexes.

### **Autophagic, lysosomal marker and mTOR**

One of the most informative marker of the lysosomal membrane is the lysosome-associated membrane protein 1 (LAMP-1)<sup>164,165</sup>. Strikingly LAMP-1

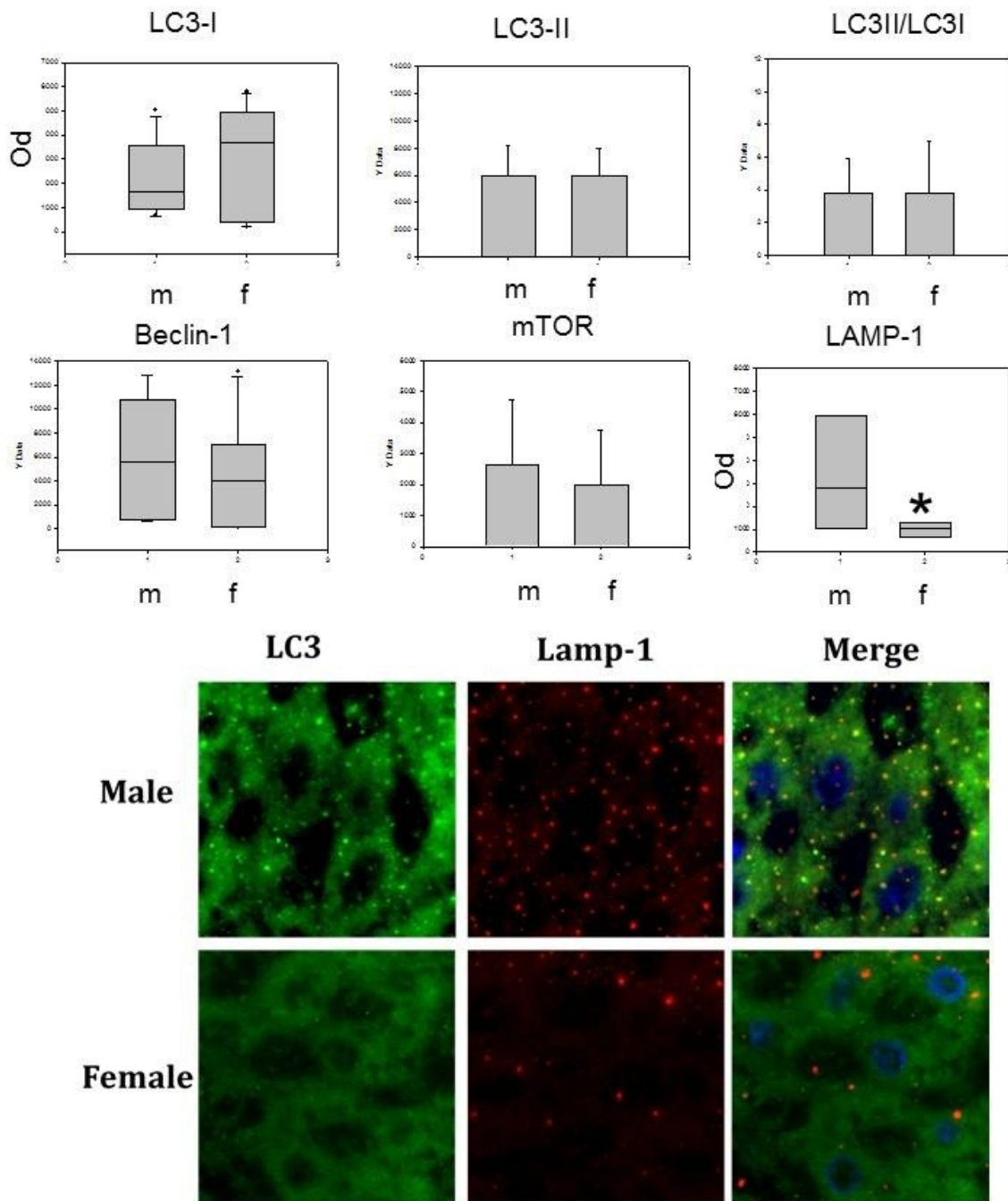


was more expressed (**Fig. 7**) in male liver than in female liver ( $P=0.04$ ). Immunofluorescence staining confirmed the datum, indeed more numerous red spots, indicative of lysosome number, were in cells of livers sections from male rats (**Fig. 7**) in comparison with those from female. Nevertheless, merging green fluorescence of LC3 immunostaining and red fluorescence of LAMP-1, a particular yellow pattern (indicative of LC3-II autophagosome accumulation) was more present in male rats (**Fig. 7**).

Expression of Beclin-1, a protein required for the initiation of the formation of the autophagosome<sup>123</sup>, and the expression of microtubule-associated protein light chain 3 (LC3), and its lipidated form obtained by conjugation to phosphatidylethanolamine (becoming LC3II) and the ratio between LC3II/LC3I did not diverge between sexes (**Fig 7**). Finally, an opposite regulator in autophagy induction the kinase mTOR, was not different in male and female livers (**Fig. 7**).

### ***Correlations between LAMP-1, beclin-1, LC3I; LC3II, mTOR***

In female livers, only one correlation has been found, in particular Beclin-1 and mTOR were directly associated (0.627;  $P=0.039$ ), in male livers none correlations was found.



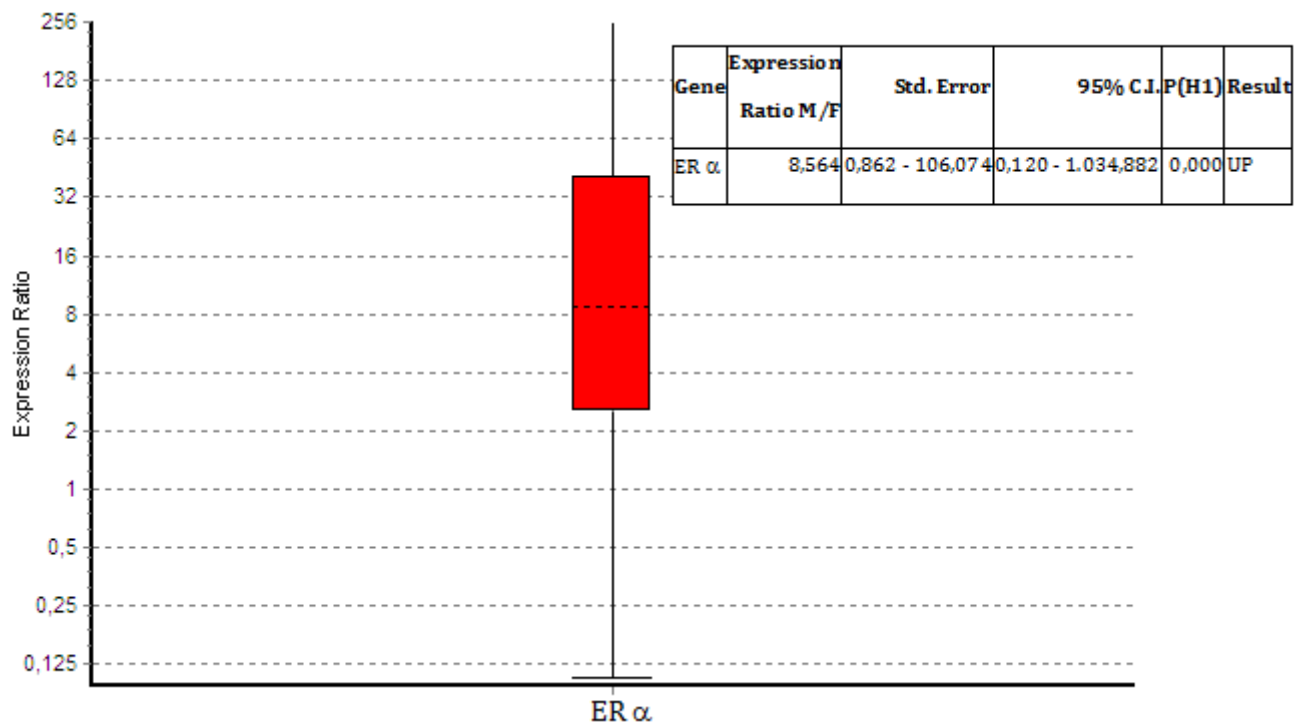
**Fig. 7.** Densitometric analysis of Western Blots of Beclin-1, mTOR, LAMP-1, LC3-I, LC3-II, LC3-II/LC3-I ratio in male and female rat livers. Data are reported as mean  $\pm$  SD or boxes with whiskers (Boxes represents the interquartile range, or the middle 50% of observations, the line represents the median; whiskers represent the 10th and 90th percentiles, dot represents the minimum and maximum observations of at least 8 independent experiments and are

normalized on total proteins \*  $P < 0.05$ . Representative immunofluorescence images of liver from a male and a female rat. Left panels show LAMP-1 positive staining (red fluorescence); middle panels show both forms of LC3 (LC3-I and LC3-II) positive staining (green fluorescence); right panels show the merge of two fluorescences (yellow punctuate pattern) indicating the co-localization of LAMP-1 and LC3.

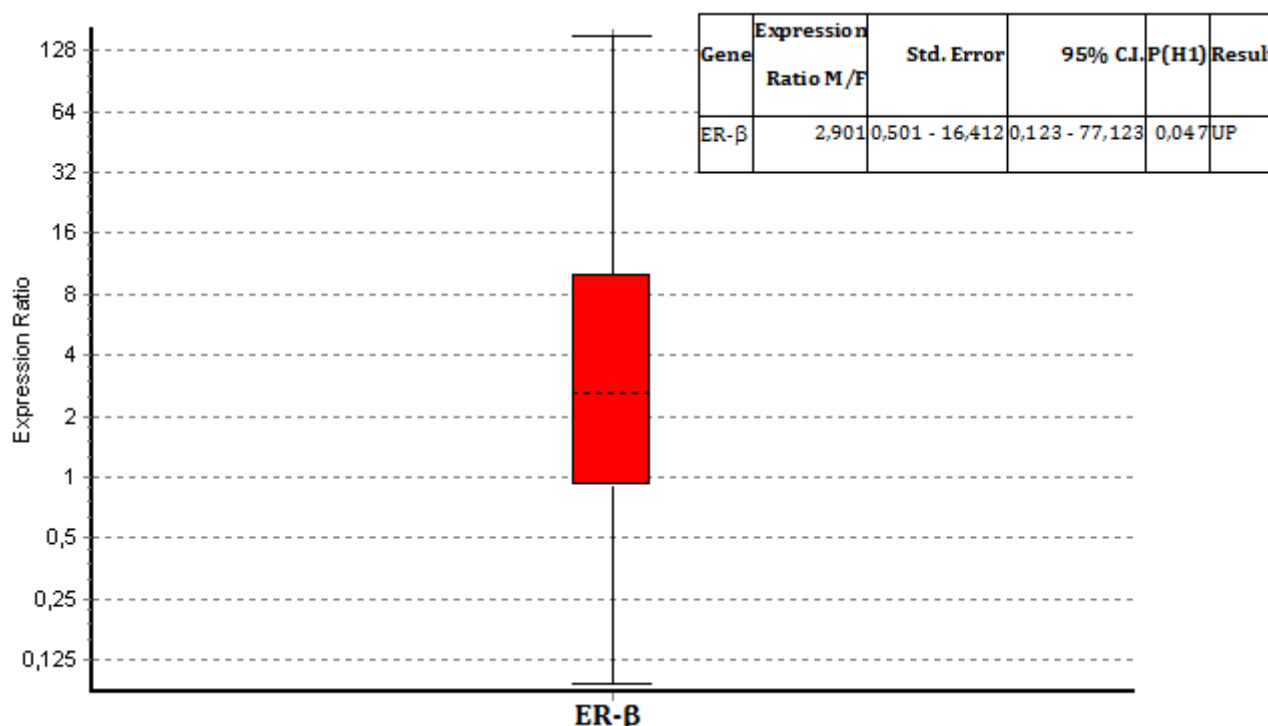
## Gene expression in HUASMC

### *Estrogen receptors alpha and beta*

Both alpha and beta estrogen nuclear receptor were markedly more expressed males than in females (Fig. 8 and 9).



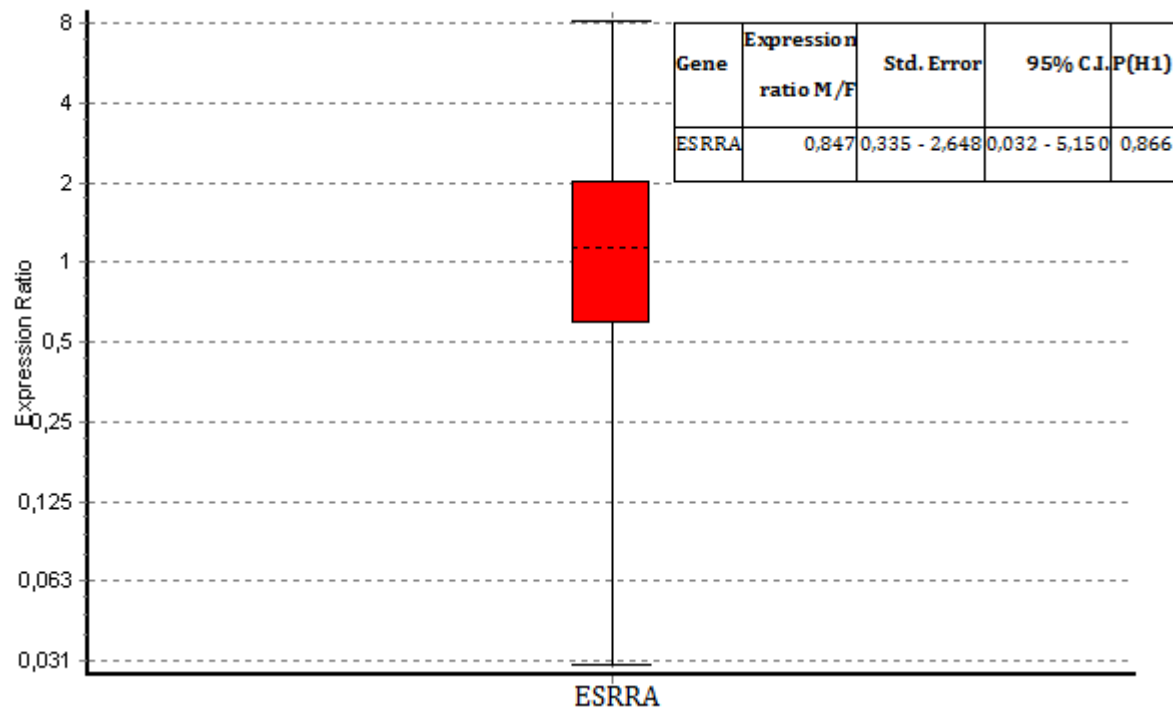
**Fig. 8.** ER alfa was up-regulated in males in comparison to females group by a mean factor of 8,564 [ $P(H1)=0,000$ ];  $P(H1)$ : probability of alternate hypothesis that difference between groups is due only to chance. Boxes represent the interquartile range, or the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations.



**Fig. 9.** ER-beta was up-regulated in male group in comparison to female group by a mean factor of 2,901 ( $P(H1)=0,047$ ). Boxes represents the interquartile range, or the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations.

### ***Estrogen-related receptor alpha***

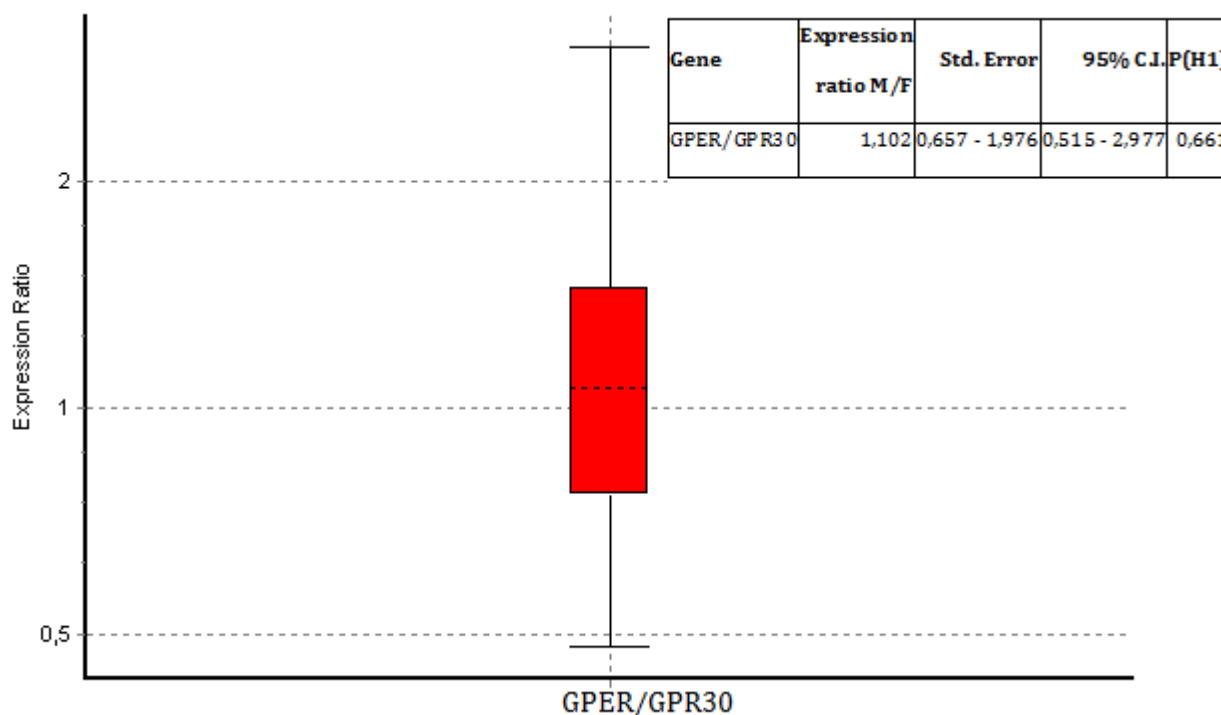
Estrogen-related receptor alpha (ERR-alpha), also known as NR3B1 (nuclear receptor subfamily 3, group B, member 1) did not diverge between sexes (**Fig. 10**).



**Fig. 10.** ESRRA expression ratio. Boxes represents the interquartile range, or the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations.

### ***G Protein coupled receptor 30***

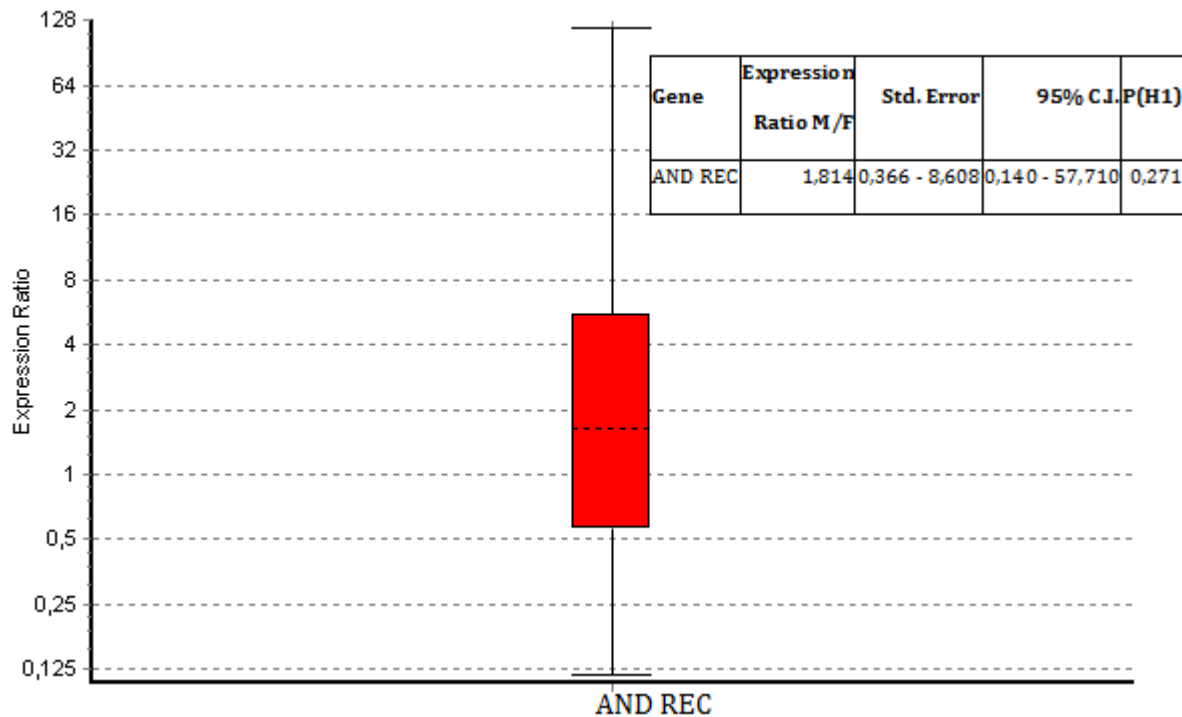
Evidence suggests that estrogen may also interact with specific membrane receptors and induce additional rapid and nongenomic vascular effects<sup>80</sup>. GRP30 as ESRRA did not diverge between sexes (**Fig. 11.**).



**Fig. 11.** GPER expression ratio. Boxes represent the interquartile range, or the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations.

### ***Androgen receptor***

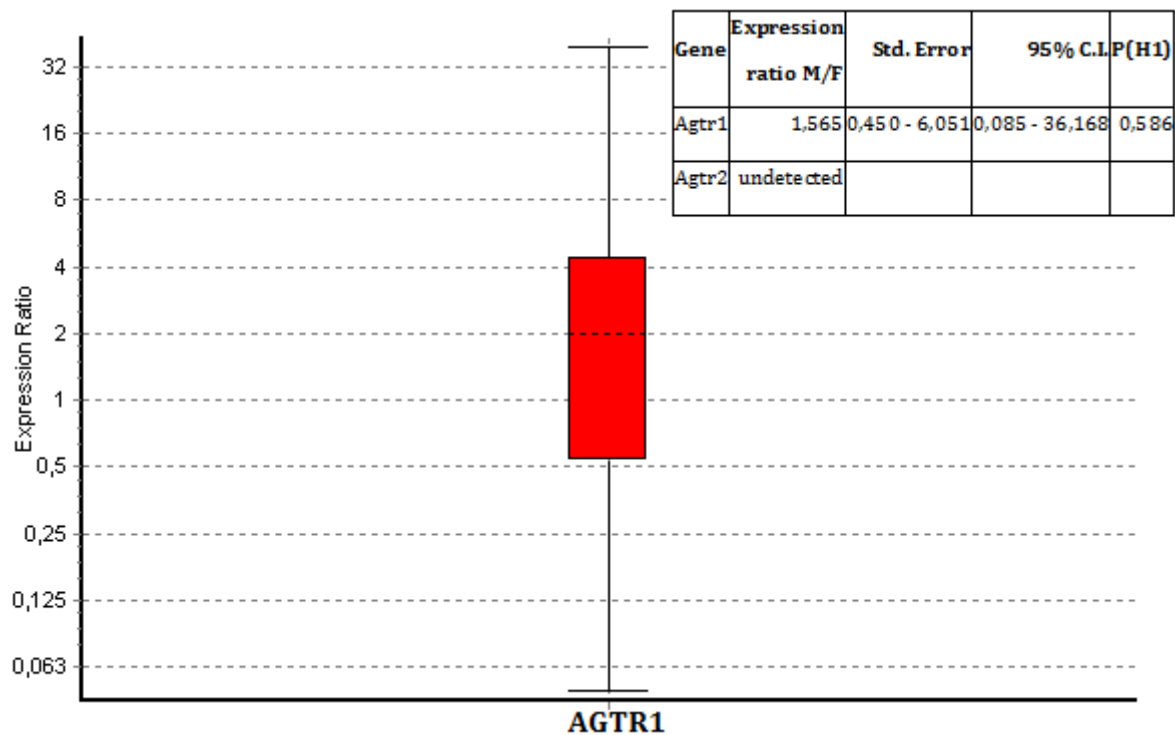
We found that male-HUASMC express more gene that encode AR than female-HUASMC but the difference was not a significant (**Fig 12**).



**Fig. 12.** Androgen receptor expression ratio. Boxes represent the interquartile range, or the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations.

### ***Ang II Receptor (AGTR) 1 and 2:***

Ang II acts through at least two types of receptors: agtr1 e 2. In HUASMC the gene that encode did no present disparity between the two sexes (**Fig.13.**), while the gene that encode AT2 receptors was undetectable. This was in line with previous results and was a further molecular confirmation that our cells cultures were free of fibroblast contamination<sup>47</sup>.

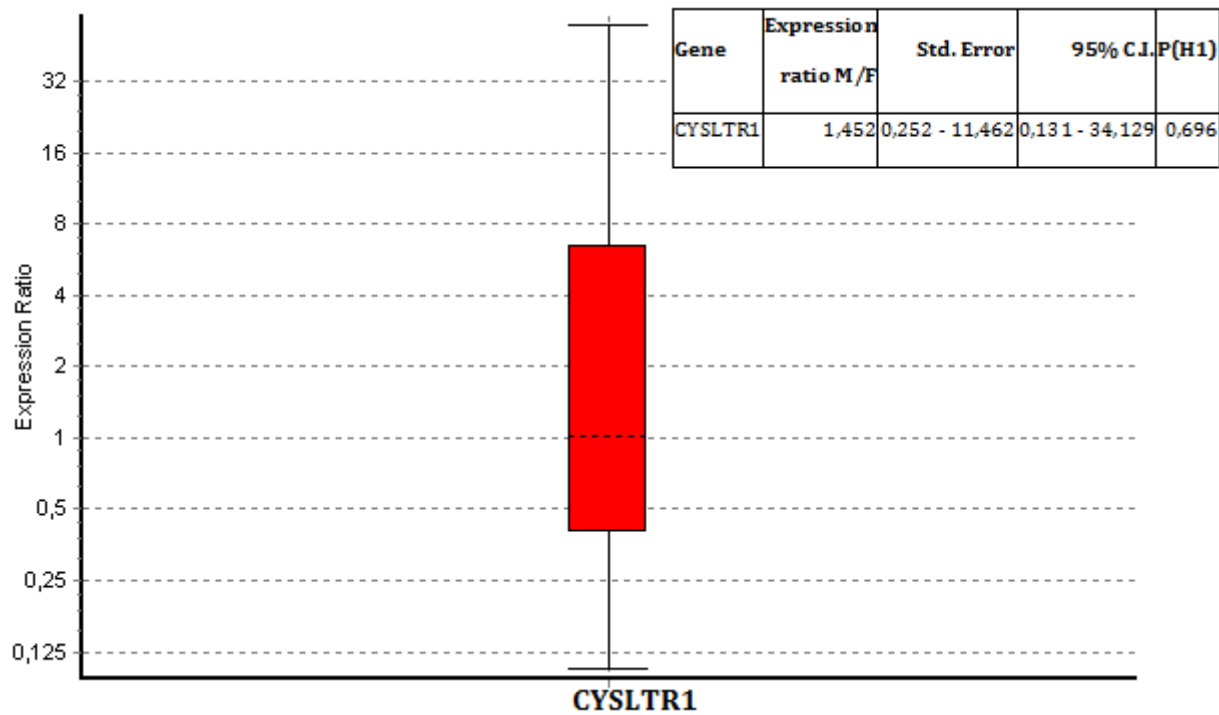


**Fig. 13.** Ang II receptor 1. Boxes represent the interquartile range, or the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations.

### ***Cysteinyl-leukotriene receptor 1***

Cysteinyl-leukotrienes are potent lipid mediators synthesized from arachidonic acid by a variety of cells and trigger contractile and inflammatory processes through the specific interaction with cell surface receptors. Cysteinyl leukotriene receptor 1 (CYSLTR1) is a member of the superfamily of G protein-coupled receptors. Activation of this receptor by LTD4 results in contraction and proliferation of VSMC. In humans CYSLTR1 gene localizes in Xq13-q21. Due to its position on the X chromosome and for its involvement in the inflammatory cascade we were wondering if it was expressed in HUASMC and most of all, if it was subjected to sex gender dimorphism or lionization (dosage compensation) in HUASMC. We found CYSLTR1 was expressed in males and females VSMC, and the difference was not significant (**Fig. 14.**).





**Fig. 14.** Cysteinyl-leukotriene receptor 1. Boxes represent the interquartile range, or the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations.

## Discussion

Numerous sexual differences has been described in the metabolic activity of the liver: one of the best known difference involves the expression and activity of hepatic drug metabolizing enzymes<sup>103</sup>. Here, we show that, glutamyl cycle, another metabolic pathway involved in drug metabolism, is sexual divergent and it is relevant in the paracetamol toxicity<sup>107,108</sup>. In particular, we found no sexual significant difference in methionine levels, the precursor of L-cysteine which in turn is the GSH precursor; but L-cysteine is higher in the female liver while GSH levels are not sexual divergent. To better understand this point, we measured the rate limiting enzyme in the synthesis of GSH that is also responsible in the early recovery of GSH. The GCL expression (measured with western blot and immunohistochemistry) is higher in the male liver than in the female one. The lower levels of GLC observed in female rat liver can impact in liver protection from xenobiotics and many reports indicate that females are more susceptible versus toxic effect of xenobiotics<sup>108,166-170</sup>. However, it is important to recall that GCL is higher in the liver of transgenic and ICR female mice than in the liver of male mice<sup>107,108,171</sup>, while in the liver of CD-1 mice, others do not find any significant differences in GCL between male and female<sup>172</sup>. The previous observations strongly suggest that the sexual disparity in GCL can be species specific as occurs for the various liver cytochrome P450 isoforms<sup>173</sup>. Indeed, our results suggest that male rats compensate for the smaller availability of L-cysteine with a major efficacy of the rate limiting step of GSH, which, as already mentioned, is higher in the male liver than in the female one.

The expression of GLC is influenced by lipid peroxidation<sup>174</sup>; however MDA, a marker of lipid peroxidation, is not different between the male and the female rat liver. Our findings counteract with previous results, which show that MDA levels were higher in the male than in the female liver<sup>175</sup>. The apparent discrepancy could be due to the dependence of MDA levels<sup>176,177</sup> on animal age. Although MDA levels are not significantly different in the male and in the female liver, we have to remark that they are higher in female livers and this could have some biological consequences. For example, it could contribute to higher L-cysteine levels observed in females, which can auto-oxidize and produce reactive oxygen species.

Finally, we examined the byproducts of L-cysteine: GSH, taurine and H<sub>2</sub>S. The two antioxidant molecules namely GSH and taurine were not significantly different. Remarkably, H<sub>2</sub>S, a molecule implicated in gastrointestinal and cardiovascular functions<sup>156-158,160,161,178</sup> is significantly higher in male liver

versus the female one. This could partially explain a major use of L-cysteine in male liver in comparison with female liver. Regarding this last point, it is important to recall that, at least in vascular tissues, testosterone influences H<sub>2</sub>S production<sup>179,180</sup>. In conclusion, GSH amounts are similar between female and male rats, while liver GCL expression in female rats is lower than in male indicating that the capacity of GSH biosynthesis to counteract the liver injury induced by various xenobiotics, may be sex-gender dependent. The importance of sex-gender difference was also confirmed by higher L-cysteine levels and lower H<sub>2</sub>S in the female liver. These results also indicate that experimental design should include male and female subjects (both animals and humans), taking into consideration methodological questions regarding sex-gender differences such as the sexual differences in housekeeping proteins.

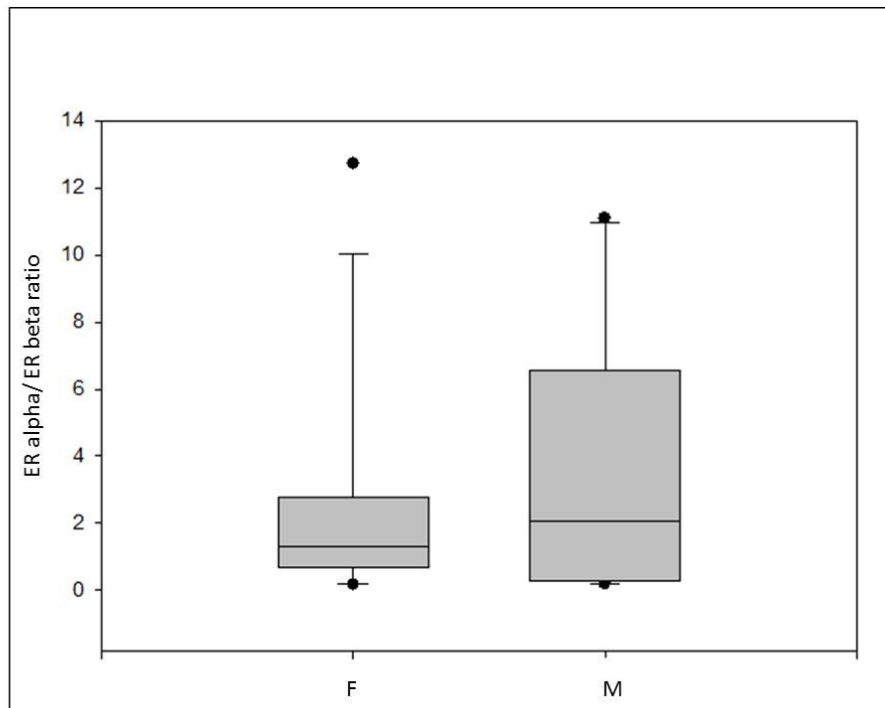
In the liver, it has been also studied autophagic process. Sex-gender differences in autophagic process have been already seen in heart of cachectic tumour-bearing mice<sup>181</sup>, in VSMC<sup>59</sup> in fibroblasts and neurons<sup>138</sup>. Basal autophagy is a conserved mechanism<sup>182</sup> for targeted degradation of superfluous or damaged organelles and is crucial to maintain cellular homeostasis and to maintain the normal structure and function of an organ or a tissue<sup>183</sup> even from the very beginning of embryogenesis<sup>124,126</sup>. Autophagy is also a way to assure cell survival or cell death. It is activated by ischemia/reperfusion<sup>131,132,184,185</sup> and by heart failure<sup>129</sup>, situations that present numerous sex-gender differences at clinical level<sup>29</sup>. The biomarker of autophagic process beclin-1, the two isoform of LC3 and their ratio, and the most important autophagic checkpoint, mTOR<sup>186,187</sup>, are not significantly different on the basis of western blot analysis. However different associations are found in male and female rat liver. In particular, a positive correlation between mTOR and beclin-1 is found in female livers but not in male ones, suggesting that in the absence of variation in protein expression, the signalling between mTOR and beclin-1 could be different in the two sexes. Considering that oxidative stress has a pivotal role in autophagy<sup>38,56,59,188</sup>, it is important to underlie that we do not observe any significant sexual variation in lipid peroxidation and protein oxidation.

Notably, either western blot or immunofluorescence of livers strongly evidence that a marker of lysosomes (LAMP-1) is significantly more expressed in male livers versus female ones indicating that the male rat liver is richer in lysosomes than the female one. LC3 and LAMP-1 colocalize suggesting that the male liver contains a greater number of autophagosomes in comparison with the female liver. Importantly, to our knowledge, this is the first report which evidences a sex-gender difference in the number of liver lysosomes and autophagosomes. Of

course in order to understand the physiological relevance of this result more studies are necessary and should include all metabolic function of lysosomes such as lipophagy<sup>189</sup>. Lipophagy regulates intracellular lipid stores and lipid metabolism and lipid metabolism presents numerous sex-gender differences<sup>190</sup>. In conclusion, although there are obvious sex-gender differences in physiology and pathology of the liver, most studies did not include male and female animals or do not analyze the results for sex, however, as shown here, when the male and females are included and analysis of results is stratified for sexes the differences emerge. Therefore, it urges to include both sexes in order to have better understanding of sex-gender differences. The consideration of the differences could short the time from bench to bed sides and could ameliorate diagnostic and therapeutic procedure in clinical practice. Importantly, the knowledge of the differences at hepatic levels considering the importance of the liver in drug metabolism could have paramount consequences in drug efficacy and safety. Adverse drug reactions (ADR) are a problem for 3~5% of subject taking a drug<sup>191-194</sup> but the frequencies in hospitalization for ADR is bigger in women than in men<sup>19,195</sup>. Women have greater risk, in comparison with men, in experiencing ADR. This risk could be reduced through the appropriate knowledge of sex and gender differences, reducing so individual, social and economic costs.

We chose HUASMC as a model for smooth muscle because they are relatively easy to obtain, although one must recall that umbilical arteries transport venous blood and live in a medium completely unique from the point of view of hormones, characterized by high levels of estrogen, progesterone and cortisol<sup>196</sup>. Despite the advantage of working on umbilical cord and the large amount of publications<sup>147,197</sup> on how isolate VSMC from their arteries, none of the methods described before has led us to a satisfactory result in terms of cell quantity, quality and purity (free from contamination from adjacent tissues), as well as simplicity, economy and reproducibility. The method that we use for isolation has been largely modified and is suitable for both female and male origin cells. In particular, as showed in the pictures (**Fig. 2-3**), both for male and female HUASMC, after only 13 days of culture p0, reached the critical density needed to induce the transition from secretory to contractile phenotype. Transition becomes definitely evident when cells reached 100% of confluency. To avoid the risk of molecular and phenotypic changes during cell culture, we proceeded to RNA isolation when cells were at first passage<sup>58,198,199</sup>. Morphology and phenotype, varies with the number of replicative cycles as an effect of telomeres shortening, leading to cellular senescence and death<sup>200</sup>.

In this experimental model we examined the expression of some genes. In particular, we measured the gene of estrogen and androgen receptors that can be involved in sex-gender differences and genes that encode receptors that play a crucial role in many function of VSMC, namely angiotensin 1 and 2 receptors and cysteinyl-leukotriene receptor 1. ESRRA, GPER30 and AR and Cysteinyl-leukotriene receptor 1 (CYSLTR1) did not diverge between sexes, while genes encoding estrogen receptor alpha and beta were sexual divergent being ERbeta and ERalpha more expressed in male and female cells, 8.5 and 3 times respectively. Consequentially, the ratio between the two receptors diverges and it might affect the percentages of the protein dimerization products (**Fig. 15**). We evaluated this hypothesis but the different ratio of ER1 and 2 gene expression between males and females is likely to be due only to chance ( $p>0.05$ ).



**Fig. 15.**  $2^{\Delta\Delta CT}$  between ER1 and ER2 representation. Boxes represents the interquartile range, or the middle 50% of observations. The line represent the median. Whiskers represent the 10th and 90th percentiles, dots represents the minimum and maximum observations.

Sex-gender differences in nuclear estrogen receptors alpha and beta gene expression are relevant because ER alpha and beta control numerous functions in many tissues<sup>201–205</sup>. For example, activated estrogen receptor-alpha in skeletal

muscle can stimulate the genomic expression even for other nuclear hormone receptors and promote long chain fatty acid uptake, mitochondrial shuttling and  $\beta$  oxidation<sup>206</sup>. Indeed, the different expression of ER1 and ER2 may be involved in the develop of many somatic tissues to say nothing of the reproductive organs<sup>207-209</sup>.

Notably, the AR receptor gene, that is located on X chromosome does not significantly diverge in male and female cells, however we found that male HUASMC express the AR gene 1.8 times more than female ones. Since the human gene encoding for the androgen receptor is located on the X chromosome (which is in two copies in female cells and in single copy in male cells), we suggest that this gene is subject to dosage compensation in order to equalize the level of gene expression between males and females<sup>112,210</sup>.

We measured also gene expression for Ang II receptors and Cysteinyl-leukotrienes receptor-1 which have a pivotal role in vascular function controlling proliferation, contraction, cytoskeletal arrangement, inflammation<sup>211-216</sup>. Nevertheless, some data suggest that renin-angiotensin system can be sexual dimorphic<sup>217</sup>. The differences could be due to different activity of AT receptors<sup>43,44</sup>, if so the difference does not depend on gene expression<sup>47</sup>. Finally, the gene which encode Cysteinyl leukotriene receptor 1 (CYSLTR1), whose activation by LTD4 results in contraction and proliferation of VSMC<sup>218</sup>, is expressed in both male and female cells and it is not sexually divergent although it is located on chromosome X.

In conclusion, the previous results suggest that sexual differences are present at the beginning of life and seem to be mainly dependent on estrogen system in comparison with androgen system. Expression levels for estrogen receptor genes are selectively regulated since differences are limited to ESR1 and ESR2.

If the reader calculates the overall percent of significant differences between two sex-gender found in this PHD thesis, it might perhaps be surprised to find that it amounts to about 38%. The occurrences observed in this study are not only statistical different between sexes. They are a biomolecular part of an amount of data indicating the necessity to pay attention to the biochemical aspects characterizing sex-gender differences in fisiopathological and pharmacological aspects. One of the lessons from the diversity found in nature is that it does not lie in the amount of DNA, but especially in the regulation of gene expression, which for some limited aspects is clearly evident, for others, often more important, must be investigated. Once this diversity comes out, it seems irrational disregard for aspects in health that are closely linked to it.

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